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Hypoxia/Reoxygenation-Induced Mutations in Mammalian Cells Detected by the Flow Cytometry Mutation Assay and Characterized by Mutant Spectrum

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Abstract

Under hypoxic conditions, cells are more resistant to cell killing by ionizing radiation by a factor of 2.5 to 3, potentially compromising the efficacy of radiotherapy. It has been shown recently that hypoxic conditions alone are sufficient to generate mutations *in vitro* and *in vivo*, likely due to the creation of reactive oxygen species (ROS) and a decrease in mismatch and homologous recombination DNA repair activity. These factors are known precursors to the onset of genetic instability and poor prognosis. We have previously characterized the flow cytometry mutation assay and its sensitivity to detect significant mutant fractions induced by genotoxic agents that are not detected by other mammalian assays. Here we measure the mutant fraction induced by hypoxia. CHO A_L cells cultured at <0.1% O₂ for 24 h generated a significant mutant fraction of 120×10^{-5} and had growth kinetics and survival characteristics similar to those obtained with other mutagens. We investigated the role of ROS by treating cells with the radical scavenger DMSO, which significantly reduced hypoxia toxicity and mutagenesis. Single cells were sorted from the mutant population, and the resulting clonal populations were stained for five antigens encoded by genes found along chromosome 11 to generate mutant spectra. The mutations were primarily large deletions, similar to those in background mutants, but the frequency was higher. We have demonstrated that hypoxic conditions alone are sufficient to generate mutations in mammalian cells in culture and that the spectrum of mutations is similar to background mutations.

INTRODUCTION

The negative effects of the hypoxic tumor environment on cancer treatment and prognosis, specifically in the area of radiotherapy, have been discussed for nearly 80 years (1,2). Decreased oxygen in the environment significantly reduces cell killing by ionizing radiation, leading to an increase in the dose required to treat the tumor successfully (3). Recently, it has been reported that hypoxic stress itself can increase mutagenesis and genetic instability in cell lines, human tissues and tumors (4).

Several pathways for hypoxia-induced mutagenesis have been proposed, including DNA damage generated by reactive oxygen species (ROS) produced by reoxygenation (5) and leakage from mitochondria (6). DNA repair activity has also been reported to decrease in

hypoxic cells and tissues, specifically in homologous recombination repair (HRR) (7,8) and mismatch repair (MMR) pathways (9). Hypoxia-induced mutagenesis and suppressed repair can lead to genetic instability and more aggressive tumors (10,11). To date hypoxia-induced mutagenesis has not been quantified using traditional mammalian mutation assays.

The use of flow cytometry to detect mutations in the *CD59* gene of CHO A_L cells has been developed by several groups independently (12–14), and we have used the flow cytometry mutation assay to measure the mutagenic potential of a broad spectrum of physical and chemical agents (15). The CHO A_L cell line was traditionally used in the CHO A_L complement cytotoxicity assay (16–18), which is based on the loss of expression of the *CD59* antigen encoded by the *CD59* gene found on the single copy of the incorporated human chromosome 11 (19). The flow cytometry mutation assay scores mutants by the absence of fluorescence when cells are stained with fluorochrome-conjugated monoclonal antibodies specific for the CD59 surface protein, while the A_L assay uses CD59-specific antibodies and complement to kill CD59⁺ cells.

We also developed a method to generate mutant spectra for clonal populations sorted from the mutant region of cells after treatment by staining for five cell surface proteins that are expressed by genes located along the entire length of human chromosome 11 (20). Mutant spectra generated in this manner are produced more rapidly than those created using PCR (21) and are equally accurate (20). Here we showed that hypoxia induces significant mutant fractions as measured by the flow cytometry and CHO A_L mutation assays. The radical scavenger DMSO increased survival and decreased mutagenesis in cells cultured under severely hypoxic conditions. We also compared the mutant spectra for background and cells kept under hypoxic conditions.

MATERIALS AND METHODS

Cell Culture

CHO A_L(N) cells have of a standard set of Chinese hamster ovary K1 chromosomes as well as a single copy of human chromosome 11, which contains a neomycin resistance gene that confers resistance to the antibiotic G418. By treating cells periodically with 800 µg/ml G418 antibiotic (Sigma-Aldrich, St. Louis, MO), spontaneous background mutants were reduced. Cells were cultured in Ham's F-12 medium supplemented with 10% FBS, penicillin/streptomycin (0.14 and 0.2 g/liter, respectively) and 7.5% (w/v) sodium bicarbonate, pH 7.3. Medium used in the CHO A_L assay was supplemented in 3% FBS and 4% newborn calf serum. Cells were maintained in T-75 tissue culture flasks at 37°C in a humidified 95% air/5% CO₂ incubator as described previously (15).

Hypoxia Treatment

A Forma Scientific water-jacketed incubator with temperature, CO₂ and oxygen control was used to obtain 2.5% to 0.1% oxygen environments. An incubator insert chamber C-274 PRO-OX Model 110 and PRO-CO2 (BioSpherix, Redfield, NY) was used to obtain oxygen levels of 0.1%. For oxygen levels <0.1% a BBL™ GasPak™ Plus Anaerobic System (BD, Sparks, MD) was used, and within 100 min of the start of the treatment oxygen levels decreased to <0.1% as measured by indicator strips included with the BBL kit. The day prior to treatment 2.5×10^5 to 4×10^5 cells were seeded in 100-mm petri dishes. Cells were plated with a minimal amount of medium to reduce the amount of oxygen present at treatment. The dishes were placed in the hypoxic system for the desired period, then placed in the incubator under normal atmospheric oxygen conditions.

Certain samples were treated with the radical scavenger dimethyl sulfoxide (DMSO). Two hours prior to hypoxia treatment DMSO was diluted (0.5% v/v) in F-12 medium and added to culture plates. Twelve hours after the end of hypoxia treatment, medium containing DMSO was replaced with fresh medium.

CHO A_L Complement Cytotoxicity Assay

After completion of hypoxia treatment, cells were cultured for 10 days to allow for recovery from the temporary growth lag as described (16,22). Cells were then challenged with 2% rabbit serum as complement (Covance Research Products Inc., Denver, PA) and 0.5% E7.1, a specific monoclonal antibody against the CD59 antigen. Surviving cells able to form colonies were counted as mutants. The mutant yield was adjusted for the plating efficiency and background mutants.

Antibody Staining for Flow Cytometry Mutation Assay

Cells (1×10^6) were trypsinized and centrifuged, and the resulting pellets were suspended in 1 ml FACS buffer (1% BSA, 0.1% sodium azide in PBS). Cells were centrifuged a second time and stained with anti-CD59 monoclonal antibodies conjugated to phycoerythrin (PE) (Caltag Laboratories, Burlingame, CA) at a 1:40 dilution in FACS and were incubated at 37°C for 1 h. One milliliter of cold FACS was then added to the cells; they were again centrifuged, the supernatant was aspirated, and the cells were finally suspended in 0.5 ml cold FACS and filtered through 40 μ m mesh into flow cytometry sample tubes on ice prior to analysis.

Multicolor Staining for Mutant Spectrum

Clonal populations were stained simultaneously for CD59, CD44 and CD90 and separately for CD151 and CD98 as reported previously (20). Cells (1×10^6) were stained with 1.25 μ l CD59-PE, 10 μ l CD90-Alexa 647, 10 μ l CD44-biotin and 28.75 μ l FACS buffer on ice for 30 min. After incubation, 1 ml FACS was added, cells were centrifuged, and the supernatant was aspirated. Samples were then stained with 1 μ l Alexa 488 Streptavidin and 99 μ l FACS and incubated on ice for 30 min. Other samples were similarly stained with 10 μ l CD98-FITC, 10 μ l CD151-PE and 30 μ l FACS for 30 min on ice. After incubation, 1 ml of buffer was added, and samples were centrifuged and resuspended in 1 ml FACS and filtered into flow cytometry tubes.

Flow Cytometry

A CyAn ADP flow cytometer with 488- and 635-nm lasers (Beckman Coulter, Fort Collins, CO) was used for quantification of mutant fractions and analysis of mutant clones. The CD59⁺ mutant regions were gated as 1% of the mean of the positive control peak on a log scale. For the mutation assay as well as clonal analysis, 1×10^5 cells from each sample were analyzed on the CyAn. Mutant regions for all other antigens were set as 97% of the negative unstained control peak, and clones were scored negative for antigens if more than 80% of the clonal population fell within the region.

Mutant Region Sorting

Samples were stained for the presence of CD59, and single CD59 negative, positive or control cells were sorted into 96-well plates containing 200 μ l F12 medium 12 days after hypoxia treatment using a MoFlo™ High-Performance Cell Sorter (Beckman Coulter, Fort Collins, CO). Cells were allowed to form colonies in 96-well plates with one refeeding. After 21 days clones were transferred to T-25 plates and expanded until there were enough cells for flow cytometry analysis of the five antigens.

Clonogenic Survival

From days 2 to 12 after hypoxia treatment, cells were trypsinized, counted and plated for survival in six-well plates. Three wells containing 4 ml medium were plated with 300 to 500 treated cells depending on the time after treatment and three wells were plated with 300 control cells. Cells were grown for 7 to 8 days before they were stained with crystal violet for colony counting. Control wells were used to calculate plating efficiency (PE), and the surviving fraction of treated cells was calculated as colonies/(PE $\times$ cells plated).

RESULTS

Hypoxia Mutant Yield in the Complement Cytotoxicity CHO A_L Assay

A survival curve was generated for cells cultured under severely hypoxic conditions (<0.1% oxygen) from 1 to 43 h (not shown), and it was determined that the time required to achieve 50% survival was 24 h. Using the CHO A_L complement cytotoxicity assay, mutant yields for cells cultured at oxygen levels of 2.5% (4 and 7 days) or 1% (4 and 7 days) were not significantly different compared to background (Fig. 1). However, cells cultured at <0.1% oxygen for 24 h demonstrated a significant induction of mutations, with 130 mutants per 1×10^5 cells.

Hypoxia Mutant Yield in the Flow Cytometry Mutation Assay

To verify these results from the cytotoxicity mutation assay, we treated cells with hypoxia and measured the mutant yield using the flow cytometry mutation assay. After a 24-h hypoxia treatment, cells were cultured for 12 days, isolated and then stained with antibodies against CD59. After staining, cells were run on a flow cytometer and the mutant region was set at 1% of the CD59-positive peak (Fig. 2) as discussed previously (13). The resulting mutant fractions are also displayed in Fig. 2. The mutant fraction from hypoxia was significantly different from controls ($P < 0.01$). The net mutant yield was 120 out of 10^5 cells after subtraction of background mutants.

Effects of DMSO on Survival and Mutagenesis

We hypothesized that damage from ROS might be causing the mutations induced by hypoxia. To test this, we treated cells with DMSO, a free radical scavenger. Cells treated concurrently with DMSO and hypoxia had reduced mutant yields in the flow cytometry mutation assay compared to cells treated with hypoxia alone (Fig. 2). DMSO had no effect on the survival of control cells but did increase the survival of cells cultured under hypoxic conditions (Fig. 3).

Survival of Mutants over Time

We have reported previously that the survival of cells sorted from the mutant regions on the peak day of mutant expression after treatment with EMS or γ radiation was lower than that of cells sorted from the CD59⁺ peak of the same population (23). The surviving fraction of cells from the CD59⁻ region (0.69 ± 0.07) was significantly less than those from the treated positive peak (0.83 ± 0.05) ($P < 0.02$). Also, the surviving fractions of cells sorted from the CD59⁺ regions of hypoxia-treated cells were lower than that of the positive peak of untreated stock cells. These results are similar to what has been found with other genotoxic agents.

We showed previously that the return to control survival of treated cells is an indicator of the peak day of mutant expression after treatment with genotoxic agents (23). Cells were sorted from hypoxia-treated and control populations from days 2–12 after treatment and grown into colonies for survival analysis. Two days after treatment, survival was only ~60% of controls, but it returned to >90% by day 12, the peak day of mutant expression for hypoxia-treated cells (data not shown).

Mutant Spectra

To determine whether hypoxia generates mostly small intragenic mutations or large multilocus mutations, individual cells were sorted for the mutant regions of hypoxia-treated cells as well as untreated control cells and grown into clones. The resulting 23 control and 25 hypoxia-treated stable clonal populations were then analyzed by flow cytometry for the presence of CD59, CD44, CD90, CD151 and CD98 on the cell surface to generate background and hypoxia-induced mutant spectra (Fig. 4).

All background mutants isolated from the mutant region of untreated control cells were negative for more than one surface protein; 17% were CD59⁺/CD90⁺, ~60% were CD59⁺/CD44⁺, and ~20% were negative for all antigens except for CD151. The mutant spectrum for clones isolated from the hypoxia-treated population was similar to that of background mutants except for a higher proportion of cells missing all markers except CD151. Of the 25 hypoxia-generated mutant clones, 4% were CD59⁺ only, 12% were CD59⁺/CD90⁺, 40% were CD59⁺/CD44⁺, 4% were CD59⁺/CD44⁺/CD98⁺, and 40% were negative for all antigens except for CD151.

DISCUSSION

DNA damage and the resulting mutations are the known precursors to cellular transformation and tumorigenesis (24). The mutagenic and carcinogenic potential of many chemical and physical agents have been characterized using mammalian cell mutation assays (15,16,25). Hypoxia decreases the mutagenic potential of ionizing radiation since the presence of oxygen helps to fix (make permanent) DNA damage (3). Nevertheless, it has been reported recently that hypoxic stress, both *in vivo* and *in vitro*, can generate DNA damage and mutations (6, 10).

Researchers have attempted to quantify the damage and resulting mutations generated by hypoxia. Hypoxia-induced DNA damage has been detected throughout the bodies of individuals exercising at high altitude and has been attributed to ROS produced upon reoxygenation (5) or to stress-induced leakage of ROS from mitochondria (6,26). Severely hypoxic conditions and the subsequent reoxygenation generate DNA damage in mammalian cells as measured using the comet assay (27). Also, hypoxia in the tumor environment decreases the activity of DNA repair mechanisms (7–9). Several groups have found up to a fourfold increase in mutations of genes located on vectors in mammalian cells grown under hypoxic conditions as well as cells grown in hypoxic tumors (10,11,28). The effects of hypoxia on mutagenesis, DNA repair and genetic instability have recently been reviewed (4).

We have demonstrated that culturing cells under hypoxic conditions for 24 h followed by reoxygenation generates a significant mutant yield as measured by the CHO A_L complement cytotoxicity assay. Using the same cell line, we have also characterized the FMCA by testing its sensitivity with agents that were known to produce only small responses or false negatives with other mammalian cell assays (15). To evaluate the frequency and type of mutations that may be induced by hypoxia, we cultured CHO A_L cells for 24 h in <0.1% oxygen and measured the mutant yield and spectrum of mutations after reoxygenation. The results from both assays clearly demonstrate that hypoxia followed by reoxygenation is sufficient to generate a significant mutant yield. This would be relevant for cycling hypoxia *in vivo*, a situation where cells might be acutely hypoxic for a period followed by reperfusion. Our results do not specifically demonstrate whether it is the reoxygenation that causes the mutations, but that is a likely possibility.

An anaerobic chamber was used to generate hypoxic culture conditions, and dry test strips indicated when oxygen levels dropped below <0.1%, which occurred within 1 h. The culture

medium and polystyrene culture dishes used during treatment contained absorbed oxygen, requiring time for the oxygen levels in the medium around the cells to equilibrate with the atmosphere in the chamber. A previous study of oxygen diffusion in polystyrene dishes demonstrated that the partial pressure of oxygen in medium (20 ml) held in 100-mm dishes reached the chamber levels within 3 h and that oxygen levels in dishes containing confluent mammalian cells dropped to 0% in under 90 min (29). From this we can be confident that oxygen levels in the medium during these experiments dropped below 0.1% in under 3 h.

We determined previously that the peak day of mutant expression falls between days 6 and 12 for 17 different mutagens (15). It is important to note that cells treated with hypoxia responded similarly in that the peak day of expression was day 12. We reported that clonogenic survival of treated cells returns to control levels at roughly the same time as the peak day of mutant expression (23). Hypoxia is no different given that the survival of the treated cells was ~90% of control on day 12. The survival of mutants sorted from the CD59⁺ region on the peak day of expression was less than that of the cells sorted from the positive peak of the same population, which again holds true with studies using known mutagens (30).

An advantage of the flow cytometry mutation assay is that mutant cells can easily be sorted and cloned to study the spectrum of mutations in different mutants. We have shown that mutants generated by γ radiation are frequently large deletions (20) while those generated by EMS are small, frequently intragenic, mutations (31). We previously compared the mutant spectrum obtained by detecting the presence or absence of CD surface proteins with the presence or absence of flanking genes obtained by PCR analysis, and there was a perfect correlation (20). Thus we can confidently say that the mutant spectra shown here do represent actual genetic mutations. Here we showed that the mutants generated by severe hypoxia were similar to spontaneous mutants isolated from control populations. Both mutant spectra typically consisted of large deletions encompassing two or more genes. Interestingly, the major difference between the spectra was that 40% of the hypoxia-generated mutants were large deletions of all markers except for CD151 while only ~20% of the background mutants had this deletion. The spontaneous mutant background is ~0.05–0.15% for the 1% mutant region. Therefore, many more mutant clones would have to be analyzed to determine whether hypoxia generates an abundance of a specific mutation.

It has been reported that hypoxic stress generates a wide range of mutations from C:G to A:T transversions to deletions (10). The base 8-oxoguanine generated by radicals upon reoxygenation is often mismatched with adenine, leading to the C:G to A:T transversions (28). Also, hypoxia significantly increases DNA strand breaks that are likely responsible for the increase in deletions (27).

Using DMSO, we attempted to demonstrate that much of the damage generated by severe hypoxia and reoxygenation was caused by oxygen radicals. Many studies have demonstrated the ability of DMSO to reduce oxygen radical-induced mutagenesis (32,33). DMSO increased the survival of hypoxia-treated cells by ~30% and decreased the mutant yield in the flow cytometry mutation assay significantly. From our results we can conclude that oxygen radicals are a significant source of DNA damage induced by hypoxic stress.

Culturing cells in an extremely hypoxic environment may generate mutations caused by ROS leakage from mitochondria and ROS generated during reoxygenation. Hypoxia in tumors can suppress repair, leading to a hypermutable phenotype that can create an environment of genetic instability (34). Along with decreased DNA repair and mutagenesis, hypoxia may no longer be just an indicator of poor prognosis for radiotherapy but may play a leading role in creating more aggressive metastases by inducing genetic instability (4).

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FIG. 1. Mutant yield (mutants/ 1×10^5 cells) using the standard CHO A_L mutation assay for cells cultured under hypoxic conditions (<0.1–2.5% oxygen) for 1 to 7 days (minimum of three experiments run in triplicate). Mutant yield is corrected for background, and the error bars represent the standard error of the mean.

FIG. 2.

Top: Example histogram of CHO A_L cells stained with PE-conjugated monoclonal antibodies against CD59 12 days after a 24-h <0.1% oxygen hypoxia treatment. Gating is set as 1% of the mean of the positive peak for hypoxia-treated cells. Bottom: Mutant yield (mutants/1 × 10⁵ cells) for control cells and cells cultured in <0.1% oxygen for 24 h. Also shown are the effects of 0.5% DMSO in F12 medium. Results are from five experiments run in triplicate (***P* < 0.01), and the error bars represent the standard error of the mean.

FIG. 3.

Survival of control cells and cells cultured for 24 h at $<0.1\%$ oxygen, then incubated in normal oxygen for 12 days. Also shown are the effects of 5% DMSO in F12 medium added 2 h before hypoxia and continued for 12 h after reoxygenation (two experiments run in triplicate, $*P < 0.05$). Error bars represent the standard error of the mean.

FIG. 4. Mutation spectra of chromosome 11 generated by flow cytometry analysis of five surface antigens for clones isolated 12 days after treatment from the 1% CD59 mutant region of (top) control cells and (bottom) hypoxia-treated cells. Missing antigens are represented by minuses while the remaining antigens are marked by pluses/shaded regions and measurements are relative to the terminus of the p-arm of chromosome 11.